Efficient Hydrogen Production Using Enzymes of the Pentose Phosphate Pathway

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Abstract

Combination of the enzymes of the pentose phosphate pathway with hydrogenase has been demonstrated to increase the hydrogen yield from glucose to nearly theoretical yield (12 H_2/1 glucose). The instability of the commercially available mesophilic enzymes as well as that of the cofactor NADP+ is an obstacle for practical application of the method to hydrogen production. Improvement of enzyme and cofactor stability was pursued by employment of thermophilic enzymes and encapsulation of the enzymes in a gas-permeable matrix. To obtain thermophilic enzymes, the pentose phosphate pathway enzymes from the thermophile *Thermotoga maritima* are being cloned and expressed in *Escherichia coli*. Primers were designed for the cloning of the target genes. The genes encoding the two NADP-dependent dehydrogenases, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were cloned into the vector PCR2.1 and expressed in *E. coli*. Production of hydrogen by enzymes in cell-free extracts of *T. maritima*, alone or in combination with *Pyrococcus furiosus* hydrogenase, was demonstrated. Studies were initiated on a novel method for practical implementation of enzymes for production of hydrogen, the incorporation of enzymes and cofactors into stable liposomes that will function as nanobioreactors. Hydrogenase and glucose dehydrogenase were encapsulated in liposomes and subsequently demonstrated to produce hydrogen.

Introduction

Need for New Methods of Hydrogen Production

The currently used commercial methods for the production of hydrogen are inadequate for utilization of hydrogen as a fuel for transportation and electricity production. These methods require energy consumption, and the reformative methods generate carbon monoxide and carbon dioxide while consuming fossil fuels such as methane or petroleum. Generation of hydrogen from renewable resources such as biomass derived from agricultural and municipal wastes would provide an alternative method with lower energy costs and would not require petroleum or methane consumption. The common anaerobic organisms found in environments such as landfills and cattle rumen produce methane and hydrogen sulfide as well as hydrogen, a gas stream less suitable for hydrogen fuel cells (Leslie, 1997).

Enzymatic Hydrogen Production

Marine anaerobic thermophiles have been characterized that produce hydrogen and carbon dioxide. The hydrogenase from one of these organisms, *Pyrococcus furiosus* (Fiala &Stetter, 1986; Egerer et al., 1982; Bryant and Adams, 1989), can be coupled *in vitro* with glucose dehydrogenase to produce hydrogen from glucose (Woodward et al., 1996; Inoue et al., 1999). Since both enzymes were chosen to use the same cofactor NADP+ (nicotinamide adenine dinucleotide phosphate, Ma et al., 1993; Ma et al., 1994), the cofactor is recycled as the glucose substrate is oxidized and molecular hydrogen is produced. This two-enzyme system produces one mol H_2 per mol glucose. When appropriate enzymes such as invertase and cellulase are added, this system can be used to produce hydrogen from biomass components such as sucrose and cellulose (Woodward et al., 1998; Woodward, Cordray, et al., 2000).

The pentose phosphate pathway (PPP) is an anabolic pathway found in most organisms. The oxidative branch of this pathway consists of two enzymes, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, that together produce 2 mol NADPH and 2 mol ribose 5-phosphate from one mol glucose 6-phosphate. The non-oxidative branch carries out one and two carbon transfers that convert pentoses to fructose 6-phosphate and glyceraldehyde 3-phosphate. Since fructose 6-phosphate is isomerized to glucose 6-phosphate by phosphohexose isomerase (glucose 6-phosphate isomerase), these sugars can be recycled back into the pathway to generate additional NADPH. By addition of hydrogenase, 12 mol H_2 per 1 mol glucose 6-phosphate can theoretically be obtained. A yield close to the theoretical value (11.6 mol H2 per 1 mol glucose 6-phosphate) was achieved by combining mesophilic PPP enzymes and *P. furiosus* hydrogenase (Woodward, Orr, et al., 2000; Fig. 1).

Thermotoga maritima as a Source of Thermophilic Enzymes

Thermotoga maritima is an anaerobic hyperthermophilic eubacterium with an optimum growth temperature of 80°C, which has been isolated from geothermally heated sea floors in Italy and the Azores. Small subunit ribosomal RNA (SSU rRNA) phylogeny places this bacterium as one of the deepest and most slowly evolving lineages in the Eubacteria (Achenbach-Richter *et al*., 1987). *T. maritima* is able to grow on many simple and complex carbohydrates including glucose, sucrose, starch, cellulose and xylan, which it ferments to lactate and acetate (Huber *et al*., 1986; Huber *et al*., 1992). This bacterium offers several advantages as a potential source of thermophilic enzymes. The bacterium does not produce H_2S. It produces several hydrolytic enzymes such as cellulases, xylanases, and invertase that are necessary for hydrolysis of biomass components to glucose and other sugars.

The complete genome has been sequenced and many of the genes identified, which greatly simplifies construction of expression subclones for recombinant protein expression (Nelson *et al*., 1999). Genes encoding the enzymes constituting the pentose phosphate pathway were identified in the completed genomic sequence. There is evidence from previous studies that these enzymes are expressed in functional form by *T. maritima*. Cell extracts of *T. maritima* have been reported to contain conventional forms of glucose 6-phosphate dehydrogenase, 2-keto-3-deoxyphosphogluconate aldolase (an enzyme of the Entner-Doudoroff Pathway) and all of the enzymes of the glycolytic (Emden-Meyerhoff) pathway (Selig *et al*., 1997). Several enzymes of interest for biomass utilization, such as cellulases, β-glucosidases, xylanases, and xylose isomerase, have also been characterized from this bacterium (Bronnenmeier et al.,1995; Vielle et al., 1995).

The *T. maritima* proteins that have been characterized resemble their mesophilic counterparts, except for their thermal stability and optimal reaction temperatures. The enzyme glyceraldehyde 3-phosphate dehydrogenase was expressed at high levels in cell extracts of *T. maritima*. Following a three-step purification, the enzyme was found to resemble its mesophilic counterparts in its properties, except for its exceptional thermostability. Sequence analysis found that thermal stability was the result of the accumulation of several small changes in amino acid sequence (Wrba et al., 1997).

Results

Design of primers for cloning of pentose phosphate genes from *T. maritima*

Production of a recombinant thermophilic pentose phosphate pathway requires the cloning and expression of a total of 10 enzymes. Therefore, data needs to be collated to locate the sequences to all these enzymes and then design primers incorporating suitable restriction sites for subsequent cloning into the expression vector, pET-15b. This information is summarized in Table 1.

Recombinant G6P Dehydrogenase and 6PG Dehydrogenase

Genomic DNA was prepared from cell pellets of *T. maritima* grown for 2 days at 70°C in N_2. Primers flanking the coding regions of the genes were used to carry out PCR (polymerase chain reaction). The PCR fragments were ligated into the plasmid vector pCR2.1. Cultures of *E. coli* strain TOP 10F' transformed with the plasmids containing the respective PPP gene were selected for ampicillin resistance and disruption of the β-galactosidase gene (Ausubel, 1990). Positive colonies were grown up in culture and insertion was confirmed by restriction digests and DNA sequencing. The pCR2.1 plasmid is not designed as an expression vector, but, due to inclusion of ribosome binding sites upstream of the gene coding regions in the PCR products (Shine & Delgarno, 1974; Shine & Delgrano, 1975), expression of the recombinant proteins was detected when log-phase cultures of the positive subclones were induced with isopropyl β–galactopyranoside. Cells were harvested after 20 hours induction and lysed with 0.1% Triton X-100, DNase and lysozyme. Host proteins were removed by heating cell lysates at 80°C for 25 min. The resulting cell extracts were then analyzed for enzyme activity (Table 2).

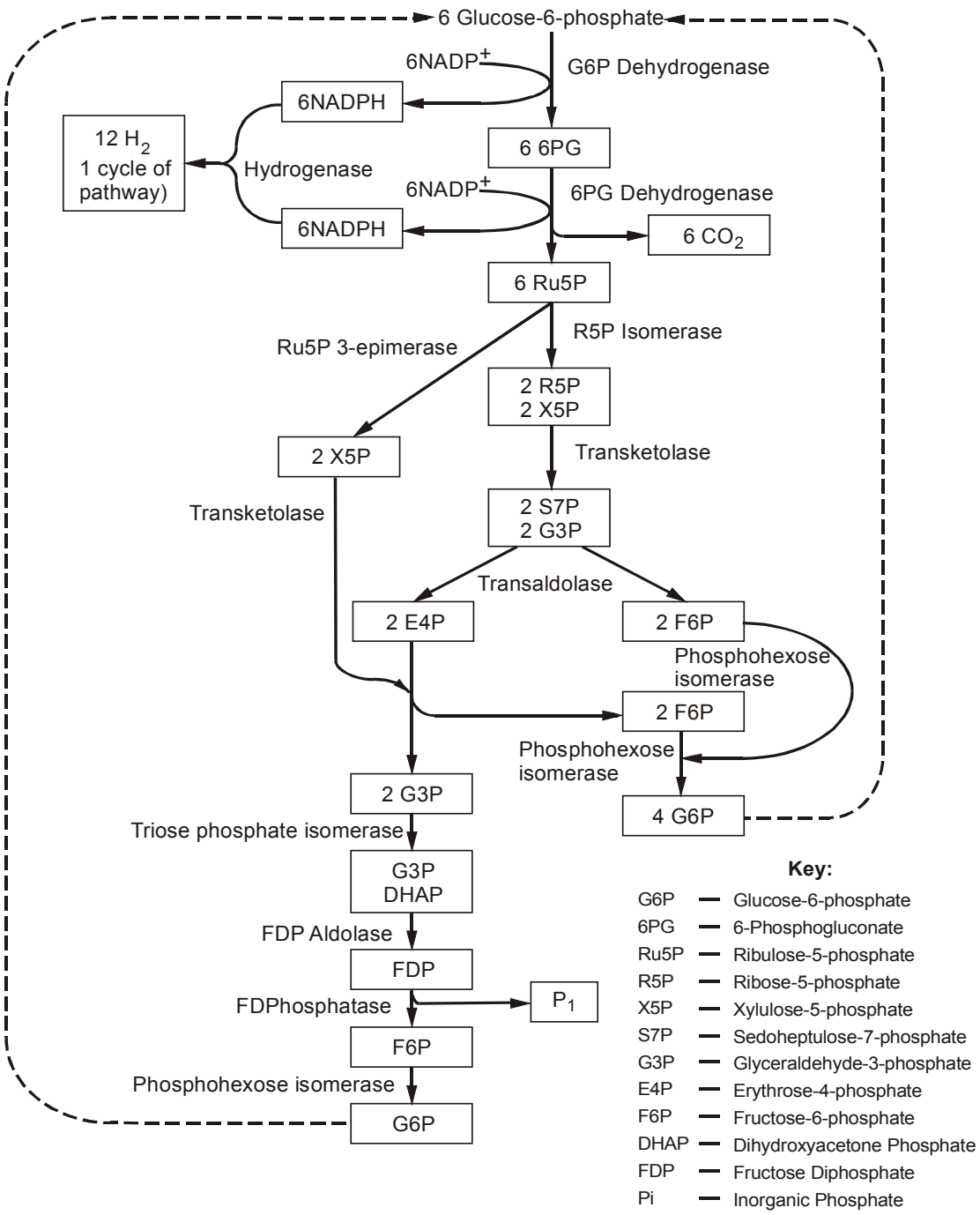

Fig. 1 Hydrogenase and the enzymes of the pentose phosphate pathway can be utilized for production of 12 mol H_2 per mol glucose.

Table 1. Location and primers for pentose phosphate enzymes in *T. maritima*.

ENZYME NAME	GENE NAME IN *T. MARITIMA* GENOME	LOCATION IN GENOME (bp)	PRIMER WITH INCORPORATED NDE I AND BPU1102 I SITES (UNDERLINED)
Glucose-6-phosphate dehydrogenase (G6PDH)	TM1155	1,168,552 - 1,170,042	5' GG CAG GTG AGC ATA TGA AGT GCA GTC 3'
			5' CCC ATC TTC GAG TCG GTA TAT C 3'
6-phosphogluconate dehydrogenase (6PGDH)	TM0438	459,482-460,891	5' GGT GAC GCA TAT GAA ATC TC 3'
			5' CCT TCG TGC TCA GCA GGT CCA CTT CG 3'
Ribose-5-phosphate isomerase (R5PI)	TM1080	1,096,137 - 1,096,568	5' GGT GCG AAG CAT ATG AAG ATC GC 3'
			5' CCT CTT CGA TTC GCA AAG G 3'
Ribulose phosphate 3-epimerase (Ru3PE)	TM1718	1,694,735 - 1,695,397	5' GGA GGA AAA ACA TAT GGT G 3'
			5' CGC AAA ATC GCT CAG CTT CAG TCA GC 3'
Transketolase (TKTlase)	TM1762	1,739,678 - 1,740,585	5' GGT GAG GTT TAC ATA TGG AAA GG 3'
			5'CCT CGT AGA AGC TTA GCA TGA ACG 3'
Transaldolase (Taldo)	TM0295	313,386-314,042	5' GGG AGG TGA ACA TAT GAA GAT C 3'
			5' GCC CGC CGA GTC GCG GGC 3'
Glucose 6-phosphate isomerase (G6PI)	TM1385	1,400,459 - 1,401,805	5' GGG GTG AA**C** ATA TGA GTT TAA AAT TCG 3'
			5' CGT TCC CTG CTC AGC GAG AGG TCC 3'
Triose phosphate isomerase (TPI)	TM0689	714,303-716,267	5' GGG GTG CGC ATA TGG AAA AAA TG 3'
			5' CCC CGC TGA GCG GGG CCT CA 3'
Fructose bisphosphate aldolase (FBPaldo)	TM0273	286,526-287,473	5' ACA TTG AAG GAC ATA TGA CAA TGC C 3'
			5' CCA GTA CGC TCA GCC TAT TTT CAC 3'
Fructose-1,6-bisphosphatase (/inositol monophosphatase) (FBPase)	TM1415	1,430,114 - 1,430,812	5' GGG GGA CAT ATG GAC AAC G 3'
			5' GAT AAT TCG ACT CGA ACA TAT CAC 3'

Table 2. Activity of *T. maritima* enzymes expressed from plasmid pCR2.1 in *E. coli*.

Sample	Weight of cells (g)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity	
					Per mg of protein	Per g of cells
G6PDH	0.9	12	2.4	48	0.05	2.6
6PGDH	0.7	12	4.8	72	0.067	6.86

Whole cell hydrogen production using *Thermotoga maritima* cultures

In order to confirm the requirement for expending resources to engineer a complete *in vitro* pathway from an organism rather than just employing the organism itself, to carry out the work, research was carried to determine the level of hydrogen that can be produced from cultures of *T. maritima*. Cultures were set up in closed vessels and grown in a standard medium (ATCC #2114) under nitrogen for 48h. The carbon sources were varied and the evolved gas was measured for levels of hydrogen and carbon dioxide using the apparatus described in Greenbaum (1984). The amounts of H_2 and CO_2 were expressed as percentage values of the amount of carbon and hydrogen potentially available in the carbon substrate used in each reaction (Table 3). Substrate utilization and total pressure in fermentation containers was not measured.

Table 3. Sugar fermentation carried out by *T. maritima* in closed vessel cultures. CMC, carboxymethyl cellulose, degree of substitution 6.5-8.5 (Sigma Chemical Co.), Avicel, microcrystalline cellulose (FMC Corp.). The amount of H_2 and CO_2 is expressed as nmol in 200 μl of head space gas.

Sugar	H_2 Produced (nmol) in 200μl of 99ml head space	CO_2 Produced (nmol) in 200μl of 99ml head space
Glucose	156.1	81
Fructose	242.53	63.19
Galactose	266.35	54.65
Mannose	9.82	187.11
Xylose	132.66	302.2
Arabinose	179.47	318.05
Lactic Acid	130.22	267.05
Gluconic Acid	112.2	241.68
Maltose	218.19	151.84
Cellobiose	162.72	309.57
Sucrose	162.11	299.04
Lactose	64.8	306.59
CMC	193.63	307.85
Avicel	62.23	37.14

Continuous Growth Culture of *T. maritima*

The culture was grown in an in-line culture vessel incorporated into the apparatus described by Greenbaum (1984). The culture was continuously purged with N_2. The overall yield of hydrogen achieved was 131.7 μmoles, which corresponds to 1.98 % of the maximal theoretical yield of H_2 that can be attained from glucose at 0.5 % in a 20 ml solution. This value is approximately 70 % greater than the identical experiment carried out in a closed vessel (Table 3).

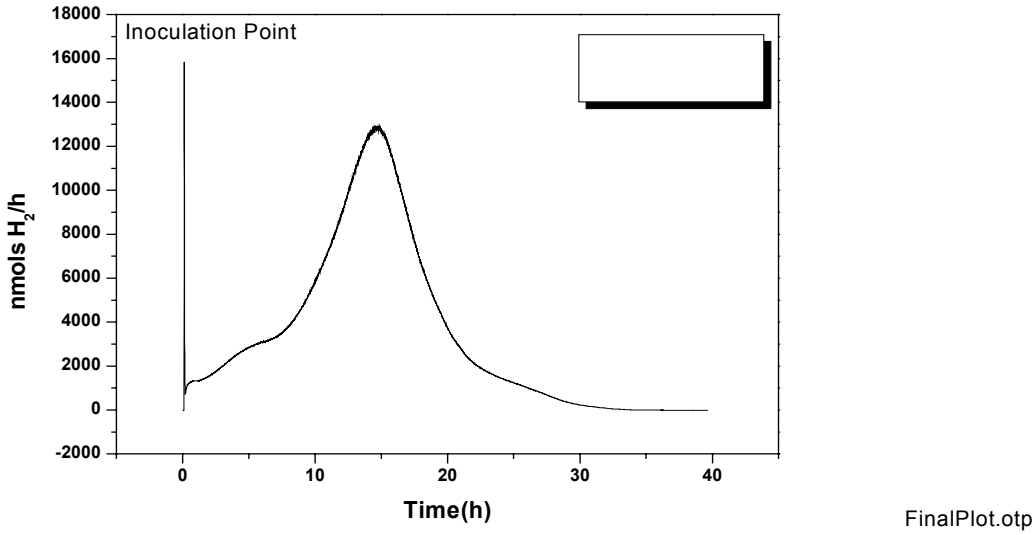

Fig 2. Hydrogen produced from 20 ml *T. maritima* culture containing 0.5% glucose at 70ºC

Hydrogen Production by Cell Extracts of *Thermotoga maritima*

The growth medium for *T. maritima* was optimized for cell growth by adjustment of the trace minerals. A yield of 390 mg/ml was achieved for the cell pellets harvested from the cultures after 48 h growth, comparable to the literature value of 400 mg/ml.

All bacterial cultures were collected by centrifugation for 1 hour at 30,000 rpm and 4°C. The growth medium supernatant was removed and the pellet was weighed. The bacterial pellet was then resuspended in 15 ml of 200 mM HEPES pH 7, passed through a French press at 30,000 psi, and centrifuged for 1 hour at 30,000 rpm at 4°C to separate cellular debris and cell extract. The cellular debris was resuspended in 15 ml of 200 mM HEPES pH 7. The activity of the key enzyme glucose 6-phosphate dehydrogenase in these fractions and the culture supernatant was assayed. The addition of protease inhibitors (BMB Complete™ Protease inhibitors) to the buffer used for the cell disruption was found to be necessary for preservation of the enzyme activity. Glucose 6-phosphate dehydrogenase activity was found mainly in the soluble fraction (88% of total activity detected), with a small amount (12%) detectable in the cellular debris. Several reaction buffers were screened, and of these HEPES was found to give the highest glucose 6-phosphate activity.

The hydrogen production capacity of the *Thermotoga* cell extract was examined as described for the continuous production by whole bacteria described above. The extract was demonstrated to produce hydrogen from glucose at 0.1μmol/hour, indicating that all enzymes and cofactors necessary for hydrogen production were present in the lysate. Addition of exogenous hydrogenase purified from *Pyrococcus furiosus* resulted in a considerable increase in the rate of hydrogen production to 1.9 μmol/hour, indicating that hydrogenase was the rate-limiting factor. The results indicate that of the *Thermotoga* enzymes necessary for production of hydrogen from glucose, only hydrogenase is oxygen-sensitive.

Enzymatic Hydrogen Production in Liposomes as Nanoscale Bioreactors

Nanoscale bioreactors made from liposomes that contain encapsulated enzymes are being designed for the enzymatic production of hydrogen from glucose (Figure 3). The enzymes and the cofactor NADP+ are retained at high local concentration inside the liposomes. The transport of glucose into liposomes is facilitated by synthetic carriers based on boronic acids (Figure 4).

Hydrogen production by a liposome-based bioreactor constructed by the encapsulation of glucose dehydrogenase from *Thermoplasma acidophilum* and *P. furiosus* hydrogenase with $NADP^+$ was demonstrated using *tert*-butylphenyl boronic acid as the glucose carrier, shown as (+) transporter in Figure 5. Encapsulation efficiency of the enzymes was 1%. Glucose was added to the exterior of liposome immediately prior to data acquisition. The control experiment shown as (-) transporter employed an identical system except the glucose carrier was omitted. Encapsulation techniques are being modified to increase the efficiency of encapsulation. A modified procedure using dried reconstituted vesicles increased the encapsulation efficiency of glucose dehydrogenase to 30 - 50%.

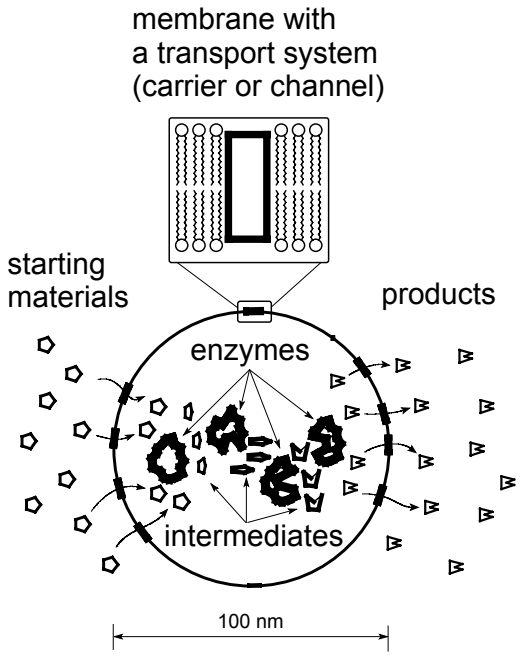

Figure 3. Concept of enzyme encapsulation in lipid bilayers to form liposome bioreactors.

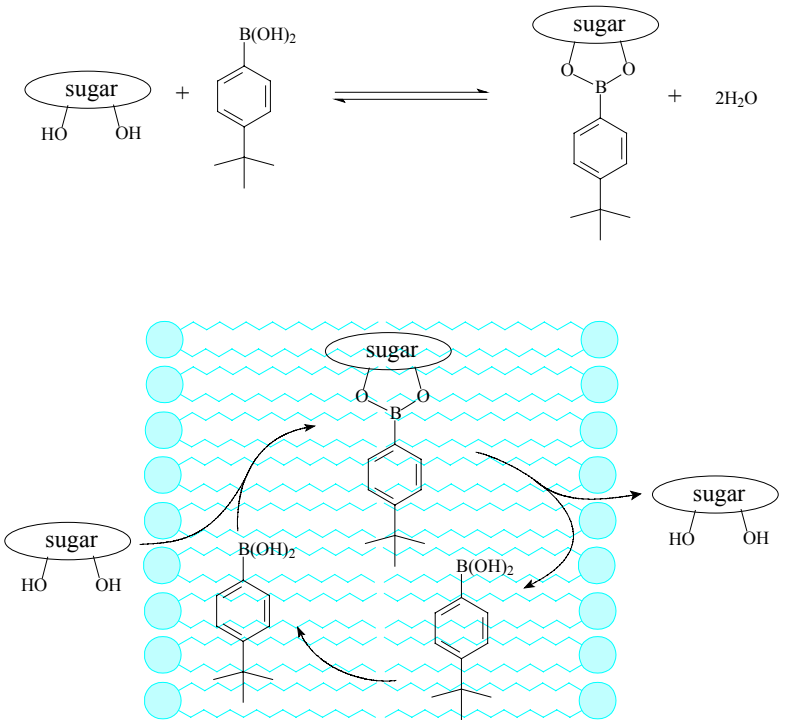

Figure 4. Transport of sugars into liposome by *tert*butylphenyl boronic acid.

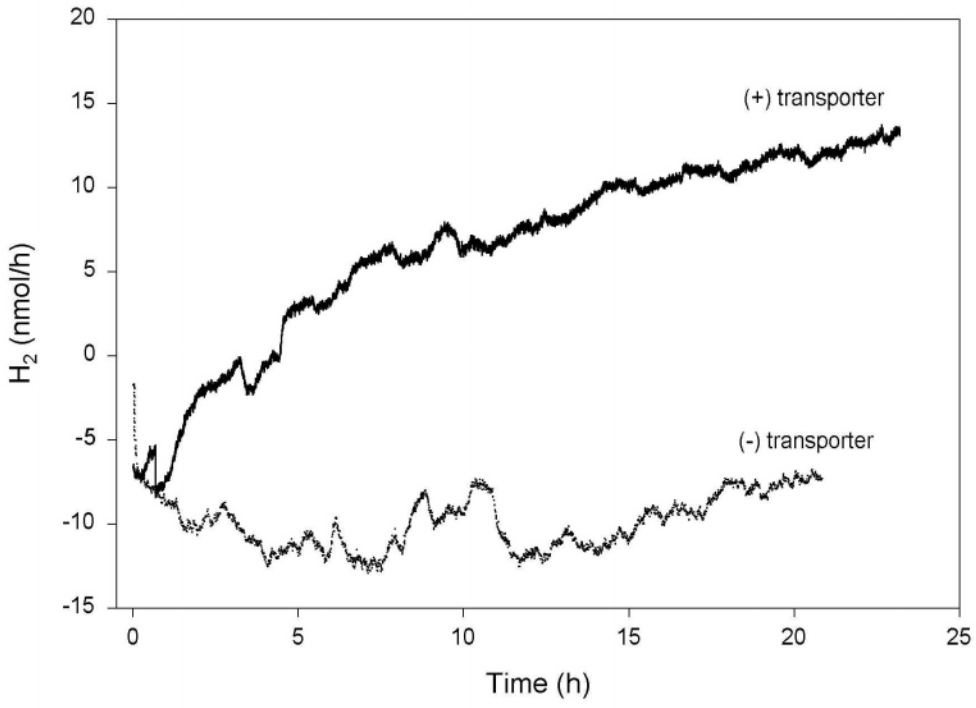

Figure 5. Production of hydrogen from glucose by encapsulated glucose dehydrogenase and hydrogenase with and without glucose transportor.

Discussion

The primers designed for PCR cloning of the pentose phosphate pathway from *T. maritima* were used to clone the two enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and expression of active proteins in the recombinant host *E. coli* was demonstrated. Improvement of enzyme yield will be attained by subcloning the gene fragments into suitable expression vector plasmids. These two enzymes are the oxidative branch of the pathway, so their incorporation into enzymatic hydrogen production reactions should theoretically increase the yield to 4 mol hydrogen/ 1 mol glucose. The appropriate primers will be used to clone the remaining enzymes for recombinant expression.

Studies of hydrogen production by *T. maritima* whole cells found that hydrogen was produced from several sugars and complex carbohydrates. This data gives insight into the metabolism of various sugars by the bacterium. For instance, glucose, fructose, and galactose are preferentially used for hydrogen production, whereas mannose metobolism mainly produces carbon dioxide. Total yield of hydrogen production was increased in a continuous flow system as opposed to a sealed culture container. However, in both systems the yields of hydrogen were low compared to the maximum theoretical yield possible. This highlights the advantage of an *in vitro* pentose phosphate pathway system in which there are no competing pathways for glucose usage. Hydrogen production from glucose was achieved *in vitro* with a *T. maritima* cell extract, and the *T. maritima* enzymes were found to be compatible with *P. furiosus* hydrogenase. This indicates that the *in vitro* system using the cloned enzymes will be successful also.

Effective application of enzymatic reactions requiring soluble cofactors such as NAD^+ and $NADP^+$ requires recycling of the cofactors and enzymes by ensuring their retention in the bioreactor by immobilization of some sort, as well as the regeneration of the cofactor (O'Neill & Woodward, 2001). Encapsulation of bioactive proteins in stable liposomes is a technique that is currently under investigation for biomedical applications (Hood et al., 2002), and approximates the natural encapsulation of enzymes in the cytosol of cells. The demonstration of hydrogen production by the encapsulated hydrogenase, glucose dehdyrogenase, and cofactor with a glucose transporter opens a new range of possible methods for design of a bioreactor containing the multiple enzymes required for the efficient production of hydrogen from glucose by the pentose phosphate pathway.

Acknowledgments

The authors would like to thank Dr Steve Blankinship and Dr Elias Greenbaum, ORNL for help and advice in carrying out the research, and Dr Frank Larimer and Mrs Shen Lu at ORNL for carrying out the sequencing. Dr. Narinder Heyer was a postdoctoral research associate through Oak Ridge Institute of Science and Engineering, Oak Ridge Associated Universities. The authors would like to thank the Hydrogen Program of the U.S. Department of Energy for funding this work. Oak Ridge National Laboratory is managed by UT-Battelle, LLC for the U.S. Department of Energy under contract DE-ACO5-00OR22725.

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